Synopsis

Reverse pharmacology approach to validate the diabetic wound-healing activity of Jatyadi thailam formulations - an *in vitro* study

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SEPTEMBER 2022

Introduction

Diabetes mellitus is a multifaceted complex disorder, characterized by hyperglycemia, that results from deregulated glucose sensing which could ultimately lead to diabetic complications. Diabetic foot ulcers are a common consequence of diabetes mellitus, with significant morbidity and mortality. In diabetics, the ability to metabolize glucose is deteriorated, which could worsen the wound healing process by perturbation of each process of wound healing, including haemostasis, inflammation, proliferation, and remodelling, resulting in a long-term deleterious impact on quality of life. Any interruption in the wound healing process has the potential to result in chronic wounds or pathological scars (Oguntibeju, 2019).

Diabetic foot ulcer is a catastrophic lower-extremity ailment caused by peripheral neurovascular insufficiency and focal infection in diabetic people. 20% of diabetics who have neuropathy have a significant risk of developing foot ulcers (Rathnayake *et al.*, 2020). A relationship between ischaemia due to atheroma, peripheral neuropathy and immunosuppression due to an accumulation of excessive glucose in the tissues, makes the body more susceptible to infection and diabetic foot ulcers (Neelambika *et al.*, 2018). In India, 4.54% of individuals with type 2 diabetes mellitus were reported to have diabetic foot ulcers; of them, 46.1 % had neuropathy, 19.7 % had ischemia, and 34.2% had neuro-ischemic foot ulcers (Das *et al.*, 2020). Diabetic foot problems pose a great threat to general healthcare professionals and national healthcare systems.

Persistent hyperglycemia and related diabetic abnormalities, such as barrier disruption and infection, increased oxidative stress, neuropathy, microvascular problems, and poor chronic inflammatory responses contribute to the complex biology of diabetic wounds. A protracted inflammatory and proliferative phase occurs in diabetic wounds which are preceded by delayed mature granulation tissue generation, reduced wound tensile strength, impaired collagen biosynthesis, and diminished angiogenesis. Ischemia-induced vascular impairment could be the cause and there are a number of factors for diabetic wounds, including peripheral neuropathy, peripheral vascular disease, and foot abnormalities (Dixon and Edmonds, 2021).

The most important components in the dynamic and complex process of wound healing are cell survival, proliferation and migration. Hyperglycemia affects keratinocytes and fibroblast biogenesis, migration, and proliferation, all of which are necessary for re-epithelialization. Endothelial dysfunction due to hyperglycemia is a major factor in diabetic

wound healing. A wound heals effectively when keratinocytes, fibroblasts, and endothelial cells migrate and respond to an inflammatory response (Burgess *et al.*, 2021).

Fibroblasts are one of the abundant cells in connective tissues, which plays an important role in tissue homeostasis, collagen and elastin synthesis. Fibroblasts are crucial from the late inflammatory phase to fully finalized epithelialized tissue in wound healing (Addis *et al.*, 2020). Different cellular communities, the extracellular matrix, and soluble mediators including growth factors and cytokines all play a role in wound healing. Diabetic wounds, do not heal normally due to an excessive accumulation of uninhibited macrophages in the chronic inflammatory stage (Wilkinson & Hardman, 2020). Therefore, some substances must be administered to the wound site to facilitate the wound healing process.

Wound care has received much focus in Ayurveda, with a focus on indigenous therapeutic procedures and the improvement of acute and chronic wound therapy treatments. Ayurvedic medications are utilised for skin wounds, especially for foot ulcers, and they serve to remove heat and toxins, increase circulation, clear blood clots, and hasten wound healing. (Dudhamal & Sonani, 2022). Ayurvedic physicians classify diabetic ulcers as Madhumeha Janya Dushtavrana. Studies have been reported on the application of Ayurvedic medicated oils locally would help to treat wounds such as diabetic wounds, clean wounds, and burger's ulcers (Shindhe *et al.,* 2020). Thus, medicated oil formulations could have a strong impact on wound healing treatment. Reverse pharmacology contributes to the scientific validation of traditional knowledge-based medicine and its use in modern medicine.

Reverse pharmacology follows the path from clinic-to-laboratory instead of the traditional ‘laboratory to clinics’ (Dayama and Patil, 2022). In India, reverse pharmacology is primarily focused on the production of standardized Ayurveda-inspired formulations, the safety and activity of which are inferred by the long and rich history of its use. For the development of medications, Ayurveda-inspired reverse pharmacology, on the other hand, provides a framework of long-term clinical experience, rationale of Ayurvedic pharmacodynamics and evidence attained via modern biomedical integration (Godse *et al.*, 2011).

The use of herbal concoctions for disease prevention and therapy has a long history. Minerals could also be added along with herbal preparations and are known as herbomineral preparations, which offer therapeutic values to diverse diseased states. These traditional

medications are usually prepared according to Pharmacopeial standards, but the mechanistic data are lacking in the scientific community (Sharma *et al.,* 2021).

Numerous ayurvedic herbal medications have been documented for their clinical benefits in reliable Ayurvedic scriptures, but their pharmacological actions and mechanisms are unexplained (Pandit *et al.,* 2022). Hence, the present study focused on the reverse pharmacology approach to validate the mechanism of action of two formulations of Jatyadi thailam in diabetic wound healing, *in vitro*.

The proposed study is one such attempt, wherein, Jatyadi thailam (JT), a popular Ayurvedic medicinal preparation used for chronic wounds was analysed using a Reverse Pharmacology approach. According to the Ayurvedic Formulary of India (AFI), the pharmacopoeia form of Jatyadi Thailam (JT-AFI) contains 16 herbs, as well as honey bee wax and copper sulphate in a sesame oil base (Mandrika *et al.,* 2021). Fascinatingly, there is another traditional version of Jatyadi thailam formulation, mentioned in Yogagrantha text (JT-YG), which is used in South India for treating chronic wounds. JT-YG contains 13 herbs which has a different composition from that of JT-AFI. JT is a popular medicated oil used to treat a variety of cutaneous wounds (Shindhe *et al.,* 2020) and excision wounds (Joshi *et al.,* 2018; Kulkarni *et al.,* 2015; Shailajan *et al.,* 2011). Despite being utilised in clinical practise for healing chronic wounds, Jatyadi thailam formulations have not been tested using modern scientific approaches.

The hypothesis set up for the present study are

Null hypothesis: Jatyadi thailam does not possess diabetic wound healing properties

Alternative hypothesis: Jatyadi thailam possesses the diabetic wound healing properties

In this study, we proposed to validate the diabetic wound healing activity of two formulations (JT-AFI and JT-YG) of Jatyadi thailam by decoding its mechanism of action in diabetic wound healing process using an *in vitro* reverse pharmacology and network pharmacology approach. The objectives framed for the present investigation were

- To assess biological/ pharmacological activity of Jatyadi thailam formulations *in vitro*
- To check the dermal toxicity of the formulations using experimental rats
- To validate the diabetic wound-healing efficacy of Jatyadi thailam formulations *in vitro* in fibroblast cell line (L929 cells)
- To elucidate the mechanism underlying in diabetic wound healing using network pharmacology approach

The current study was designed to investigate the pharmacological activity of JT-AFI and JT-YG by checking its free radical scavenging activity, biomolecule protective effects of thailam on membrane lipids, antimicrobial and anti-inflammatory activities *in vitro.* We analyzed the dermal safety of JT-AFI and JT-YG on rats by skin irritation potential and sub-acute toxicity testing. We then investigated the diabetic wound-healing efficacy of JT-AFI and JT-YG on L929 mouse fibroblast cell line, where the proliferation, migration and angiogenic potential of the formulations were studied. Furthermore, regulation of cell cycle analysis, the extent of cell death, and ROS generation were examined using flow cytometry. We then adopted a reverse pharmacophore mapping and network pharmacology approach to look into the potential mechanism of action and pathways involved in the diabetic wound healing process.

In order to attain these objectives, investigations were carried out in four phases.

Solubility

One of the key factors in reaching the desired drug concentration in the systemic circulation and the necessary pharmacological action is solubility. Since both the formulations of Jatyadi thailam are an oil-based formulations, an ideal solubilisation system to meet the aqueous-organic barrier in order to deliver the components of the thailam to the test system is imperative. Various solvents in the order of increasing polarity, such as hexane, diethyl ether, ethyl acetate, chloroform, acetone, DMSO, ethanol, methanol, water and hydroethanol was tested. Absolute solubility was not attained for both the formulations of Jatyadi thailam. To resolve this problem, we attempted to use PEG-40 hydrogenated castor oil (PEG-40 HCO), a non-ionic surfactant in oral, topical, and parenteral drug delivery systems. This was utilized as a non-ionic oil-in-water solubilizer and the solubility was improved. Further, to avoid emulsification, SPAN 80 was used, which facilitated the accessibility to transdermal delivery system. Therefore, a combination mixture of PEG-40 HCO, SPAN 80 and water, which resulted in complete solubility was used for further studies.

Phase I

In the first phase, the pharmacological activity of JT-AFI and JT-YG formulations were evaluated by assessing the free radical scavenging activity, biomolecule protective effect of thailam on membrane lipids, antimicrobial and anti-inflammatory activity *invitro.* Dose range of JT-AFI and JT-YG was optimized as 500µg/ml to 2500µg/ml by DPPH, ABTS, H_2O_2, OH•, NO and superoxide radical scavenging activity.

Jatyadi thailam was tested using a battery of free radical assays namely, DPPH, ABTS, H_2O_2, $OH^{\cdot}$, $NO^{\cdot}$ and $O_2^{\bullet-}$ radical scavenging activity, reducing power assay, total antioxidant potential by Ferric thiocyanate (FTC) method and FRAP assay. In all these methods, both JT-AFI and JT-YG scavenged the DPPH, $ABTS^{\cdot+}$, H_2O_2, $OH^{\cdot}$, $NO^{\cdot}$ and $O_2^{\bullet-}$ radicals effectively in a concentration dependent manner. The various plant components in the formulations may transfer electrons to the radicals and neutralise the reactivity of free radicals, thereby allowing the free radicals to be scavenged. This maybe the reason for the radical scavenging ability of these Jatyadi thailam formulations. The reducing power assay determines the antioxidant potential of Jatyadi thailam to transform Fe^{3+} to Fe^{2+} state, which was found to be in increasing order with increasing dose. Antioxidant activity of JT-AFI and JT-YG using ferric thiocyanate revealed that JT-AFI and JT-YG possessed significant antioxidant potential when compared to positive control ascorbic acid ($P<0.05$). Thus, Jatyadi thailam possessed antioxidant ability by suppressing the lipid peroxide production in a linoleic acid system. In Ferric Reducing Antioxidant Potential (FRAP) assay, the interaction of the antioxidant component of Jatyadi thailam formulations with the Fe^{3+} complex, were able to reduce the colourless Fe^{3+}-TPTZ complex into intense blue Fe^{2+}-TPTZ. This results in the reduction of ferric ion, which denotes the strong reducing ability of JT-AFI and JT-YG. In all these methods, higher free radical scavenging and antioxidant activity was noted in JT-AFI. The quantified free radical scavenging activity showed different inhibition rates, which might be due to the distinct reaction of antioxidant components in the Jatyadi thailam with the certain radicals or oxidants (Chaves, 2020).

Biomolecular protective effects of JT-AFI and JT-YG was studied using three distinct membrane models, namely, goat RBC ghosts (plasma membrane lipids), goat liver homogenate (plasma membrane and intracellular lipids), and liver slices (intact cells) to measure the inhibition of lipid peroxidation process. Both the formulations showed its maximum inhibition in the goat liver homogenate followed by the RBC ghosts and liver slices and maximum protection was rendered by JT-AFI. Despite the fact that all three systems contain plasma membrane lipids, the experimental data revealed that a stronger level of protection occurs in liver homogenate than in plasma membrane and intact cells. This implies that cells endogenous component operates in tandem with the Jatyadi thailam components to provide enhanced protection against lipid degradation.

Antimicrobial activity by Microbroth dilution method was employed to determine the minimum inhibitory concentrations (MICs) and minimum bactericidal and minimum fungicidal concentrations (MBCs/MFCs) of JT-AFI and JT-YG. Both JT-AFI and JT-YG

exhibited great bacteriostatic and bactericidal effect against all the tested gram negative (*E. coli, P. aeruginosa and K. pneumoniae*) and gram-positive strains (*B. subtilis and S. aureus*). Among all the tested strains, *Staphylococcus aureus* was found to have least MIC value. According to reports, *S. aureus* is the most prevalent isolate (76%) in diabetic wounds and foot ulcers, which affects the wound healing rate as well as its virulence and toxicogenic effects are strongly linked to diabetic foot infections (Goldstein *et al.,* 1996; Dunyach-Remy *et al.,* 2016). This authenticates the antibacterial activity of JT-AFI and JT-YG against the common gram-positive *S. aureus* and other wound infection causing pathogens. Similar to antibacterial activity, JT-AFI exhibited effective fungicidal activity than JT-YG on all tested fungal strains with little higher activity against *A. fumigatus.* This suggests that JT-AFI may contain rich bioactive substances that could improve the antimicrobial function by lowering the MIC value. Thus, JT-AFI exhibited better bactericidal and fungicidal activity, which was observed from the MBC and MFC values. The phytocompounds in the Jatyadi thailam formulations may probably inhibit the microorganisms through disruption in bacterial cell membrane permeability and thus acts as a promising antimicrobial formulation against skin and wound infections.

Anti-inflammatory activity was studied in cell free-system, human red blood cell membrane (HRBC) as well as using RAW264.7 macrophage cells. Protein denaturation assay revealed that the inhibition rate gradually increased with concentration and significantly higher inhibition was shown by JT-AFI than JT-YG. The findings of membrane stabilization and heat-induced hemolysis using human red blood cell membrane implied that JT-AFI and JT-YG stabilized the membrane of RBC by offering protection to the cells by suppressing the intracellular efflux against the hypotonicity as well as heat-induced hemolysis in a dose-dependent manner. Proteinase inhibition assay again authenticated our results of higher anti-inflammatory potential of JT-AFI by offering protection against proteinase action and hence prevent tissue damage. The components of Jatyadi thailam may promote wound-healing by inhibiting protein denaturation and proteinase action, thereby preventing inflammatory response.

Following the analysis of Jatyadi thailam in cell-free system, the anti-inflammatory activity was confirmed by using RAW 264.7 macrophage cells. Initially, cytotoxicity was assessed using the MTT assay to check the cell viability of the formulations. MTT results indicated that JT-AFI and JT-YG has no deleterious effect on the viability of RAW264.7 cells at the tested concentrations (cell viability≥80%). RAW 264.7 macrophages exposed to LPS, produced nitrite, which implied NO production. Our results showed that NO production was

significantly inhibited in presence of JT-AFI and JT-YG in a dose-dependent manner. JT-AFI exhibited much higher potency than JT-YG at 2500µg/ml. The standard diclofenac showed a drop in nitrite level, exhibiting its normal anti-inflammatory response. Interestingly, JT-AFI was observed to be a better NO suppressor than JT-YG. Thus, these results provide evidence of Jatyadi thailam, being more pharmaceutically beneficial in the management of inflammatory process.

Inhibition of 5-Lipoxygenase (5-LOX) and Cyclooxygenase enzymes (COX-1 and COX-2) is crucial in determining the anti-inflammatory activity of drugs. From the results of the 5-LOX experiment, it was found that JT-AFI and JT-YG significantly inhibited 5-lipoxygenase in a dose-dependent manner, with JT-AFI showing the strongest activity with the highest percentage inhibition of 5-LOX enzyme. The study also showed that both JT-AFI and JT-YG suppressed COX enzyme effectively in a concentration dependent manner. JT-AFI hindered COX-1 and COX-2 enzyme activity with the highest inhibition and had greater selectivity for COX- 2, whereas inhibition by JT-YG was COX-1 selective. Thus, JT-AFI and JT-YG are capable of diminishing leukotriene and prostaglandin synthesis and completely limits inflammation, while JT-AFI was found to be a better COX inhibitor, when compared with JT-YG. The standard drugs, such as nordihydroguaiaretic acid and Indomethacin are effective against the COX and 5-LOX enzymes. These results strengthened the anti-inflammatory actions on the 5-LOX and COX enzymes. Thus, Jatyadi thailam may therefore function as a dual inhibitor of the 5-LOX and COX and act as a potent anti-inflammatory formulation in chronic wound healing.

Phase I results clearly indicated that JT-AFI had higher therapeutic efficacy than JT-YG in combating the infection and inflammatory conditions. These observations have significant implications, as free radicals and inflammation at the wound site of non-healing diabetic wounds can interfere and confound with the wound-healing treatment measures.

Phase II

Since, our study is a reverse pharmacology approach of validating Jatyadi thailam, which is already in use in diabetic wound healing, wherein we intended to scientifically confirm the dermal efficacy of JT-AFI and JT-YG in experimental animals. Our investigation is mainly hypothesized on *in vitro* studies, only primary skin irritation potential and subacute dermal toxicity experiments were employed in this phase of work to confirm the efficacy of topical use of these selected ayurvedic formulations.

According to OECD guidelines, female rats are used in acute toxicity studies and male rats were used in the sub-acute dermal toxicity tests. In acute dermal toxicity, a total of 12 female rats were divided into 4 groups (n=3) and exposed to test formulations for 14 days. For sub-acute dermal toxicity, a total 12 male rats were divided into 4 groups (n=3) namely, G1, G2, G3 and G4, and each treatment group was applied with the JT-AFI and JT-YG once, daily for 28 days. The treatment group setup was:

G1 group: 1000mg/kg bodyweight of JT-AFI

G2 group: 2000mg/kg bodyweight of JT-AFI

G3 group: 1000mg/kg bodyweight of JT-YG

G4 group: 2000mg/kg bodyweight of JT-YG

Left side of each rat served as its own control site, where distilled water was applied.

Skin irritation test: To verify dermal reactions to the formulations, fur was removed and the test substance was smeared onto this site. The skin reaction at the application site was subjectively recorded and rated at 1, 24, 48, 72 hours, 7 and 14 days (post-test observation period) after the patch removal. After the stipulated 24 hr time period of the application of JT-AFI and JT-YG on test sites, no erythema's and edema were observed in any of the treated groups and the score for skin irritation in rats was observed to be '0'. No animal indicated edema or erythema formation after the application of formulations. Even at the end of 72 hrs of exposure, none of the animals showed either the erythema or edema, indicating the primary irritation to be '0' for both JT-AFI and JT-YG treated groups and thus, found to be non-irritant to the skin. No mortality was reported during the study. According to OECD guidelines, female rats are considered to be more sensitive to toxicity than male rats. In our study, both the formulations did not exhibit any mortality or skin irritation in female rats, thus supports the acceptability of the formulations for dermal applications.

Sub-acute dermal toxicity: JT-AFI and JT-YG was applied daily to the skin of male rats in a dose of 2000mg/kg and 1000mg/kg body weight, one dose per group and observed for 28days. After the application of single dose of 2000mg/kg and 1000mg/kg body weight of JT-AFI and JT-YG each, the rats became less active during the first hour. This might be due to the anxiety developed while shaving and covering of skin by gauze before applying the formulations. The animals treated with JT-AFI and JT-YG were found to be healthy and did not display any critical anomalies on skin and fur, breathing, debilitation in nourishment admission and water utilization. Motor activity, grip strength and sensory stimuli of the rats in all the treated groups appeared to be normal. The animals survived well during the entire

treatment period of 28days. No lethal signs and mortality were reported in our study indicating the safety of these formulations as topical applicants.

Thus, Jatyadi thailam formulations were non-irritant to rat skin in single and repeated application of doses. Hence, phase II data confirms the dermal toxicity characteristics of Jatyadi thailam formulations and hence, determining the human safe dose for long-term topical use.

Phase III

The third phase of the study pertains to unravel the wound healing mechanism of Jatyadi thailam formulations using L929 cell by analyzing fibroblast proliferation, migration and angiogenesis. We preceeded by treating the fibroblast cell line L929 with various concentrations of JT-AFI and JT-YG at different time intervals to optimize the dose and time period. For the purpose of simulating diabetic environment *in vitro,* cells were exposed to 1mM normal glucose (normoglycemic) and 50mM high-glucose media. The treatment groups set up were a) Cells alone cultured in 1mM normal glucose medium (normoglycemic) b) Cells cultured in 50mM high glucose media (Cells +HG) c) Cells cultured in high glucose media with JT-AFI (JT-AFI+HG) d) Cells cultured in high glucose media with JT-YG (JT-YG+HG). JT-AFI and JT-YG were taken in 5 different concentrations ranging from 500µg/ml to 2500µg/ml at four different time intervals (12hours, 24hours, 48hours and 96 hours) and tested on L929 cells cultured in high glucose condition in order to optimize the dose and exposure time.

The outcome of dose optimization by MTT revealed that the IC_{50} value was found to be 1000µg/ml for JT-AFI and 1500µg/ml for JT-YG, with a 24-hour exposure time being the optimum. Further research was conducted with this IC_{50} value. With this fixed dosage, the cell viability was again tested at 5 different concentrations ranging from 62.5 µg/ml to 1000 µg/ml for JT-AFI and from 93.75 µg/ml to 1500 µg/ml for JT-YG using MTT formazan. No significant cytotoxicity was measured in JT-AFI+HG and JT-YG+HG treated cells. Hence, we could infer that JT-AFI and JT-YG maintained the viability of HG-treated fibroblasts cells and could be assessed for further wound healing studies.

Cell proliferation and migration are two fundamental events crucial for wound healing and an essential event during re-epithelialization. Fibroblast proliferation is crucial in determining the wound healing ability to provoke tissue regeneration. The cell proliferation study is carried out in JT-AFI+HG and JT-YG+HG treated cells with the gradient concentrations ranging from 62.5 to 1000 µg/ml for JT-AFI and 93.75 µg/ml to 1500 µg/ml for JT-YG up to 96hr time period. The results showed increments in cell number of the treated

groups over a 72hr time period, as determined by MTS assay. Similarly, the results of SRB assay again affirmed that JT-AFI and JT-YG increased the cell proliferation under high glucose condition, indicating the cell viability.

Angiogenic property was assessed by a simple, accurate and sensitive method of *in vitro* tube formation, which is important in wound-healing process. Fibroblasts contribute to connective tissue development by extracellular matrix deposition and promotes capillary formation and stabilization through production of angiogenic factors in close vicinity to endothelial cells. The cells were cultured on matrigel and treated with fixed doses of JT-AFI+HG and JT-YG+HG and tube formation were recorded at different time intervals such as, 2^{nd} hr, 4^{th} hr, 6^{th} hr, 8^{th} hr, 10^{th} hr, 22^{nd} hr and 26^{th} hr. The formation of tube-like structures increased when the cells were treated with JT-AFI and JT-YG during 26hr time period. The number of branches were also significantly increased in the JT-AFI treated cells than JT-YG and HG treated cells. This might be due to the release of more angiogenic factors by Jatyadi thailam formulations.

Migration of fibroblasts are pivotal in understanding the primary stage of wound healing process. This was studied by scratch assay using L929 cells at different time intervals- 0^{th} hour, 6^{th} hour, 12^{th} hour, 24^{th} hour and 48^{th} hour. The exposure of cells to JT-AFI+HG and JT-YG+HG significantly increased the number of migrating cells towards the wound-site from 12^{th} hour. The cells treated with HG showed lesser number of migratory cells, when compared with control cells. Thus, during the 48hour observation period, the wound closure time was faster in cells exposed to JT-AFI from 12^{th} hour itself.

Angiogenesis describes the development of new capillaries from pre-existing blood in the vicinity of the wound as capillary-like formations. CAM assay was employed to find out the performance of Jatyadi thailam formulations to induce microvascular process. In our investigation, control group indicated pattern for normal angiogenesis, whereas, the blood vessel formation in chick embryo treated with high glucose was significantly reduced, indicating delayed or anti-angiogenic potential. On the contrary, CAM treated with JT-AFI was angiogenic in terms of increase in vessel density and branching points. JT-YG did not exhibit any angiogenic response after 72hours of treatment. Thus, angiogenic property of Jatyadi thailam formulations was evident, which highlighted on the increase in blood vessels by JT-AFI indicating its active and effective angiogenesis activity in chick embryo model.

Colony formation assay measures cells proliferative integrity, or its long-term survival ability. To further confirm the promotion of proliferative ability of the cells by JT-AFI and JT-YG, we performed colony formation assay in L929 cells. JT-AFI+HG and JT-YG+HG

encouraged the clonogenic potential of cells at its fixed doses, where it showed a greater number of anchorage-dependent colonies. However, JT-AFI showed significantly higher proliferative capacity with an increase in colony number than JT-YG treated cells. HG treated cells showed a reduction in colony number when compared to other treatment groups. This describes the cell proliferation inducing ability by Jatyadi thailam towards healing of diabetic wounds.

In diabetic wound healing studies, it is crucial to understand the cellular responses during therapeutic interventions. Hence, the changes involved in cell cycle, apoptosis and ROS generation in the process of diabetic wound-healing was probed. Proliferating cells sequentially transit through the G1-S-G2-M phases to prepare for DNA synthesis, cell division, and the ensuing mitotic process as the cell cycle progresses. Cell cycle analysis revealed that the percentage of cells in G2/M phase significantly increased in HG alone treated cells when compared to control. The cells treated with JT-AFI+HG and JT-YG+HG significantly decreased G2/M phase cells compared with HG group, but stimulated them to enter the S phase by increasing the proportion of cells at G1/G0 phase. This result indicates a decreased number of arrested cells in the mitotic phase; a result that shows a cell proliferation is aided by Jatyadi thailam. JT-AFI appears to be more active in stimulating cell proliferation, when the distribution of cells in each stage of the cell cycle is taken into account.

Analyzing cell death mechanism is important to understand the modulatory effects of Jatyadi thailam in apoptosis and necrosis. The cells were stained with Annexin V-FITC/PI and examined by flow cytometry to evaluate the mechanism of apoptotic events. The proportion of apoptotic cells were high in HG treated group compared to control cells, where no noticeable change was observed. In high glucose environment, JT-AFI and JT-YG relatively reduced the extent of apoptosis. A slight increase in necrosis was found in JT-AFI treated cells. Although necrosis triggers an inflammatory reaction, the inflammatory stage is a crucial and preliminary step in the formation of new cells during wound healing therapy. We can infer that a minimal increase in necrosis would not have a detrimental impact on the wound healing process. As necrotic cells have an influence on cell migration and invasion and thus, an increase in necrosis might not impact the wound healing process (Ahn *et al*., 2016). This can also be related to ROS, because an excess of ROS causes oxidative stress, which in turn causes apoptosis (Hu *et al.*, 2014). This decrease in apoptosis in our study may be attributed to the antioxidant activity of Jatyadi thailam that facilitates the clearance of free radicals and regulates apoptosis.

Chronic wounds are brought on by ROS and further leads to devastating complications. We used 2,7-dichlorofluorescein diacetate fluorescent staining to determine the influence of

active concentrations of Jatyadi thailam to HG-induced ROS generation in fibroblasts. A marked increase in intracellular ROS generation was observed in HG-treated cells than control cells. JT-AFI and JT-YG are able to counteract the ROS production in high glucose treated cells. These findings suggest that Jatyadi thailam formulations mitigated oxidative stress in HG-stimulated fibroblasts, which in turn denotes a greater antioxidant effect. Consistent with these reports, our results demonstrated that, despite both Jatyadi thailam formulations having adequate ROS attenuating properties, JT-AFI treated cells act as a great ROS scavenger.

From the above results, it was evident that the *in vitro* study demonstrated that both JT-AFI and JT-YG promotes the healing of diabetic wounds by accelerating the fibroblast cell proliferation, migration and angiogenesis. Interestingly, the results of flow cytometry analysis supported the *in vitro* results and also authenticates the mechanistic role of Jatyadi thailam towards diabetic wound healing by aiding the cell proliferation/survival, regulating cell cycle events, alleviating the DNA damage and ROS generation on diabetic mimic cell line model.

Consistent with our previous phase results, JT-AFI had more beneficial effect towards chronic wounds than JT-YG, which might be due to the variations in the exotic medicinal plants and bioactive components. This could cause the difference in therapeutic efficacy towards diabetic wound healing.

Phase IV

In light of these findings, JT-AFI was observed to have a better wound healing efficacy in diabetic wounds. Therefore, in this phase of the study, JT-AFI alone was selected. In this phase of work, we adopted network pharmacology approach, which involved reverse screening of phytocompounds and its related targets in diabetic wound healing to discover its mechanism of action.

Jatyadi thailam contains 16 herbs with a sesame oil base along with beewax and copper sulphate. Beewax and copper sulphate is used as a base in ayurvedic formulation. A total of 1229 phytocompounds from 16 herbs were retrieved from plant databases, such as Indian Medicinal Plants, Phytochemistry and Therapeutics (IMPPAT), Phytochemical Interactions Database (PCIDB) and Duke phytochemical database. Out of the 1229 compounds, 120 compounds met the criteria of drug likeliness (DL) ≥ 0.18. Hence, these 120 compounds were considered to be the key active compounds responsible for the therapeutic effect of Jatyadi thailam. The highest number of compounds were found in *Glycyrrhiza glabra* (55 active compounds) followed by 19 active compounds in *Prunus cerasoides,* 15 active compounds in

Azadirachta indica, 7 active compounds in *Pongamia pinnata*, 5 active compounds in *Berberis aristata.*

In order to identify the potential targets of the compounds, target fishing was done using PharmMapper 2018 database as well as disease databases, namely Gene Cards and comparative toxicogenomics database (CTD). We screened for effective targets (Homosapiens) for all 120 compounds using PharmMapper database, which identified 17 targets corresponding to the onset and healing of diabetic wounds. 7373 and 17087 targets were retrieved from Gene card and CTD databases related to diabetic wounds. Once the targets were retrieved from these individual databases, we wanted to identify the key targets common to all these databases. Hence, integration of these targets was done using venny tool, which identified 14 key targets.

Protein-protein interaction (PPI) network between the target proteins comprises of 14 nodes and 11 edges; each edge represents PPIs. The other parameter is average node degree with a value of 1.57, which corresponds to the average number of interactions (at the score threshold) that the proteins have in this network. The average local clustering co-efficient is 0.448, which measures the nodes network connectivity. In this PPI network, among 14 putative targets, four targets, namely PGF, TGFBR1, FOLH1, PPARD were not found to interact with other targets. But they are reported to have a contributing role towards onset and healing of diabetic wounds. Hence, all the 14 targets were taken into account for further studies.

Compound-target interaction network system was constructed to understand the interaction between the compounds of Jatyadi thailam and the putative targets. The 120 active compounds were attributed to 14 putative targets with 314 edges. In this network, the active compounds of *G. glabra* were able to interact with more number of targets compared to other active compounds. We observed that the targets in this network interacted with multiple compounds of Jatyadi thailam. Thus, the active biochemical compounds of Jatyadi thailam might have a synergistic effect on these targets of diabetic wounds. In this network, based on the degree of nodes, we found the compounds (-)-Epicatechin Gallate and Wistin was connected to all targets with the highest degree of 69, followed by Dihydrooroxylin, Quercetin (32 degrees), ()-Nimocinolide, Zafaral (29 degrees), Eupatin, Ponganone iii (21 degrees), (.+/.)-Karachine, Palmatine (18 degrees) interacts with multiple targets of diabetic wounds, thereby exerting its pharmacological effects in enhancing the healing of diabetic wounds.

"Compound-target-pathway" network construction was done to gain insights on the potential mechanism of Jatyadi thailam on diabetic wound-healing. The Compound-target-pathway network demonstrated the relationship in multiple pathways inclusive of the transit interaction and cross-talk pathway There are 161 nodes (137 herb-compound nodes, 14 disease-

related target nodes and 10 pathway nodes) and 336 edges in Jatyadi thailam-disease-pathway network. Therefore, the therapeutic property of Jatyadi thailam components in diabetic foot ulcers were uncovered through multi-components, multi-target, multi-pathway interactions, which may jointly regulate the healing process of diabetic wounds.

The target information was analyzed with GO enrichment and KEGG pathway annotation. It focussed on the revelation of relationship network of these putative targets through biological function, cellular component and molecular function. Based on GO annotation and KEGG enrichment analysis, the targets corresponding to JT-AFI interfered with the cellular proliferation through diversified functional and signaling pathways. The GO terms "collagen biosynthetic process", "muscle cell proliferation", "response to endogenous stimulus" were closely related to wound healing process. It is an indication that, targets involved in these pathways could recruit fibroblast cells and boosts fibroblasts to produce collagen and also could aid angiogenesis through muscle cell proliferation. Mast cell granule, nuclear transcription factor complex, RNA polymerase II transcription, nuclear receptor transcription coactivator activity, RNA polymerase II repressing transcription factor binding and dipeptidase activity were the terms enriched in cellular component and molecular function.

KEGG enrichment analysis showed that the putative targets were enriched in multiple pathways, such as the Chagas disease, AGE-RAGE signaling pathway in diabetic complications, Phenylalanine, tyrosine and tryptophan biosynthesis, Complement and coagulation cascades, Biosynthesis of amino acids, PPAR signaling pathway, Pathways in cancer, Pentose and glucuronate interconversions, Fructose and mannose metabolism, Folate biosynthesis (hsa00790). AGE-RAGE signaling pathway (hsa04933) was reported to the key pathway in diabetic complications. Other enriched pathways could play a role in regulation of carbohydrate metabolism and helps to improve glucose uptake, thereby controlling the diabetic complications.

This AGE-RAGE is a multiligand transmembrane receptor, which plays a pivotal role in chronic inflammatory conditions. Binding of AGE to RAGE activates NF-κB signaling pathway and causes tissue damage, marking the important role in pathogenesis of diabetic complications. In our study, among the 14 key targets, 3 targets such as TGFBR1, PRKCB and SERPINE 1 were found to be involved in AGE-RAGE signaling pathway.

In order to explicate whether JT-AFI could have an effect on the expression of these targets, western blotting was performed with L929 cells. TGFβ plays a critical role in the successful migration, angiogenesis and granulation tissue formation. This TGFβ mediates it signaling through TGFβ receptor I (TGFβRI). In chronic non-healing wounds, this TGFβ

pathway is suppressed, which inturn, failed to epithelialize the wound (Ramirez *et al.,* 2014). Upon AGE-RAGE binding, RAGE triggers the downstream signaling cascade through PKC. PKC is calcium dependent and under high glucose condition in endothelial cells, PRKCB inhibits PI3K/AKT-dependent nitric oxide synthase regulation by insulin, leading to endothelial dysfunction and also oxidative stress in hyperglycemia mediate the vascular dysfunction (Koya and King, 1998). SERPINE 1 (also termed plasminogen activator inhibitor 1 or PAI-1) is a well-known TGFβ target gene and is an activator of TGFβ signaling pathways that are stimulated in response to wounding. SERPINE1 mediates epithelial wound healing through urokinase plasminogen activator and was reported to modulate migration, angiogenesis and cellular adhesion. Upregulation of SERPINE1 has been reported to cause wound healing in cornea in diabetic patients (Sun *et al.,* 2015). Thus, our result of Immunoblots showed that the proteins TGFRB1 and SERPINE1 are overexpressed and the expression of PRKCB is suppressed in a time-dependent manner (6hr, 12hr, 24hr and 48hr) in presence of JT-AFI treated cells, when compared to the untreated cells. β-actin expression was utilized as a control. The investigation gave us an insight that these three targets may mitigate the diabetic wound healing process of JT-AFI, thereby mediating the regulation of AGE-RAGE signaling pathway in overcoming the diabetic complication, such as diabetic wounds.

To sum up phase IV, the main active ingredients in the treatment of diabetic foot ulcers by JT-AFI are (-)-Epicatechin Gallate and Wistin, which corresponded to *Glycyrrhiza glabra.* Collagen biosynthetic process, muscle cell proliferation, regulation of cell proliferation, RNA polymerase II repressing transcription factor binding, AGE-RAGE signaling pathway are important signaling pathways involved in diabetic foot ulcer or diabetic complications. JT-AFI can play a role in the treatment of diabetic foot ulcers through multiple targets-multiple pathways network. This shows that controlling one or more network targets can control the entire network, giving Jatyadi thailam, a scientific basis for treating diabetic foot ulcers.

To conclude, these positive findings could provide scientific evidence for using Jatyadi thailam in treating non-healing foot ulcers by combating the infection and inflammatory mediators at the wound-site. The three targets involved in the AGE-RAGE signaling pathway can be probed further to better understand the physiology behind the diabetic wound-healing process.

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